RESEARCH ARTICLE

A preliminary molecular phylogeny of the genus *Scobura*, with a synonym of *Scobura masutaroi* (Lepidoptera, Hesperiidae)

Zhen-Fu Huang[1,*], Wen Fei[1,*], Min Wang[1], Hideyuki Chiba[2], Xiao-Ling Fan[1]

1 *Department of Entomology, College of Ariculture, South China Agricultural University, Guangzhou 510642, China* **2** *B. P. Bishop Museum, 1525 Bernice Street, Honolulu, Hawaii, 96817-0916 U.S.A.*

Corresponding authors: *Xiao-Ling Fan* (fanxiaol66@scau.edu.cn); *Hideyuki Chiba* (skipper@i.bekkoame.ne.jp)

Citation: Huang Z-F, Fei W, Wang M, Chiba H, Fan X-L (2016) A preliminary molecular phylogeny of the genus *Scobura*, with a synonym of *Scobura masutaroi* (Lepidoptera, Hesperiidae). ZooKeys 638: 33–44. https://doi.org/10.3897/zookeys.638.10026

Abstract

A molecular phylogeny of the genus *Scobura* based on the mitochondrial COI and the nuclear EF-1α genes using maximum likelihood and Bayesian inference is proposed. The analyses include 19 specimens from nine ingroup species. The monophyly of *Scobura* is not strongly supported, but two strongly supported monophyletic groups within the genus are recognized: the *S. coniata* group and the *S. woolletti* group. Judging from combination of the molecular evidence and morphological features, the former consists of six species, including *S. masutaroi*, while four species belong to the latter. *S. mouchai* Krajcik, 2013 is confirmed to be a **syn. n.** of *S. masutaroi* Sugiyama, 1996. The key to the species of the genus *Scobura* is modified to reflect these results.

Keywords

COI, EF-1α, *Scobura masutaroi*, *Scobura mouchai*

---

* These authors contributed equally to this work and should be considered co-first authors.

Introduction

The skipper genus *Scobura* Elwes & Edwards, 1897 was recently revised by Fan et al. (2010), who recognized 14 species. The genus *Scobura*, however, includes another species, *S. masutaroi*, Sugiyama 1996. Fan et al. (2010) overlooked the existence of this taxon and did not include it in their revisional work, which resulted in Krajcik (2013) proposing a new taxon, *S. mouchai*, from Shaanxi.

Although a comprehensive morphological revision of the genus has been completed, no phylogenetic analysis has been performed to infer relationships within the genus. In the present study, we present a preliminary phylogeny of *Scobura*, based on molecular evidence. By comparing molecular and morphological evidence, we examine whether *S. mouchai* is a synonym of *S. masutaroi*.

Methods

Morphological examination

See Fan et al. (2010) for materials for the morphological study. In order to examine the wing venation, wings were removed from thorax, cleaned with 95% ethanol, and dyed red with acetocarmine (Wang et al. 2011).

Taxon sampling

Twenty-three specimens including nine of the 15 valid species of *Scobura* and four outgroup species were included in the phylogenetic reconstruction. Detailed information on the specimens is provided in Table 1. Specimens used in this study were mainly deposited in the Insect Collection, Department of Entomology, South China Agriculture University (SCAU), except for some specimens in Kyushu University museum (KU) and Mr. Hiroaki Onodera's private collection.

Laboratory protocols

Genomic DNA was extracted from the thorax of specimens preserved in ethanol, or from legs of dried specimens, using Magen's Blood/cell/tissue DNA extraction kit. One mitochondrial gene *cytochrome c oxidase I* (COI) and one nuclear gene *elongation factor 1-α* (EF-1α) were used as molecular phylogenetic markers. The following primers were used for amplification and sequencing in this study: for COI – primers LCO1490 and HCO2198 (Folmer et al. 1994); for EF-1α – primers ef44 and efrcM4 (Monteiro and Pierce 2001). Ploymerase Chain Reaction (PCR) were performed in 20 μl volumes containing 1 μl

Table 1. Voucher information and GenBank accession numbers for the specimens in this study.

Species	Locality	Latitude	Longitude	Voucher Number	COI	EF-1α
*Scobura cephaloides kinka*Evans, 1949	China: Hainan	19.02N	109.53E	SCAU He102	KY049936	KY049958
*Scobura cephaloides kinka*Evans, 1949	Laos: Luang Prabang	19.93N	102.07E	Onodera He553	KY049937	KY049959
Scobura coniata Hering, 1918	China: Guangdong	24.91N	113.04E	SCAU He073	KY049938	KY049960
Scobura coniata Hering, 1918	China: Guangdong	24.87N	113.03E	SCAU He472	KY049939	KY049961
Scobura hainana (Gu & Wang, 1997)	China: Guangdong	24.87N	113.04E	SCAU He471	KY049940	KY049962
Scobura hainana (Gu & Wang, 1997)	China: Guangdong	24.87N	113.04E	SCAU He487	KY049941	KY049963
Scobura hainana (Gu & Wang, 1997)	China: Guangdong	24.87N	113.04E	SCAU He488	KY049942	KY049964
Scobura isota (Swinhoe, 1893)	Thailand: Kanchanaburi	14.08N	99.36E	SCAU He538	KY049943	KY049965
Scobura isota (Swinhoe, 1893)	Thailand: Mae Hong Son	19.35N	98.14E	SCAU He468	KY049944	KY049966
Scobura lyso (Evans, 1939)	China: Zhejiang	30.15N	119.25E	SCAU He465	KY049945	—
Scobura lyso (Evans, 1939)	China: Zhejiang	30.15N	119.25E	SCAU He475	KY049946	—
Scobura masutaroi Sugiyama, 1996	China: Sichuan	29.94N	102.48E	SCAU He300	KY049947	KY049967
Scobura masutaroi Sugiyama, 1996	China: Sichuan	29.94N	102.48E	SCAU He301	KY049948	KY049968
Scobura masutaroi Sugiyama, 1996 (=*mouchai*)	China: Shaanxi	31.91N	106.34E	SCAU He303	KY049949	KY049969
Scobura parawoolletti Fan et al., 2010	China: Hainan	19.03N	109.53E	SCAU He116	KY049950	KY049970
Scobura stellata Fan et al., 2010	China: Guangdong	24.92N	113.01E	SCAU He036	KY049951	KY049971
Scobura woolletti (Riley, 1923)	Indonesia: Kabandungan	6.77 S	106.60E	KU He535	KY049952	KY049972
Scobura woolletti (Riley, 1923)	Indonesia: Kabandungan	6.77 S	106.60E	KU He536	KY049953	KY049973
Scobura woolletti (Riley, 1923)	Indonesia: Kabandungan	6.77 S	106.60E	KU He537	KY049954	KY049974
Suastus gremius (Fabricius, 1798)	China: Guangdong	23.15N	113.34E	SCAU He157	KY049955	KY049975
Suada swerga (deNicéville, 1884)	Thailand: Chiang Mai	18.80N	98.92E	SCAU He495	KY049956	KY049976
Hyarotis quinquepunctatus Fan & Chiba, 2008	China: Hainan	19.03N	109.54E	SCAU He114	—	KY049977
Zographetus satwa (deNicéville, 1884)	China: Guangdong	24.88N	113.03E	SCAU He442	KY049957	KY049978

template DNA, 2 µl 10× buffer, 1.6 µl dNTPs (containing 2.5 mM of each dNTP), 0.8 µl of each primer (10 uM), 0.2 µl Taq Polymerase (2 U/µl), and 13.6 µl ddH$_2$O. The PCR Products were amplified using initial denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 47 °C (COI) for 45 s, 55 °C (EF-1α) for 1 min, elongation at 72 °C for 1.5 min, and final elongation at 72 °C for 5 min.

Amplified DNA products were purified using an Agarose Gel Extraction kit (Magen Biotech), and directly sequenced, or cloned with pMD18-T vector (Takara Inc), and then sequenced. Sequencing was performed using the ABI 3730 automated sequencer. All sequences were submitted to the Genbank database (accession numbers are given in Table1).

Phylogenetic analyses

Alignment of the DNA sequences were performed in Clustal X (Thompson et al. 1997) and edited manually in MEGA 6.0 (Tamura et al. 2013). All base frequencies and molecular character statistics were calculated in MEGA 6.0. Phylogenetic trees were constructed under maximum Likelihood (ML) and Bayesian inference (BI) criteria. For ML analysis, RAxML version 8 (Stamatakis et al. 2014) was used on a concatenated data set of two genes, with 1000 rapid bootstrap replicates using GTR+G substitution model on the CIPRES Science Gateway (Miller et al. 2010). BI was carried out using Markov Chain Monte Carlo (MCMC) randomization in MrBayes v3.2.3 (Ronquist et al. 2012). We used reversible-jump MCMC to allow for sampling across the entire substitution rate models. Four Markov chains (three heated chains, one cold) were run for 500, 000 generations, with the first 25% of sampled trees discarded as burn-in. The two independent runs were considered to have converged when the standard deviation of split frequencies value was <0.01. The convergence of the analysis was determined in Tracer v1.6 (Rambaut et al. 2014). Bayesian posterior probabilities (PP) and ML bootstrap values (BP) were used to evaluate branch support.

Results

Sequence data

From a total of 23 samples, 22 sequences for COI and 21 for EF-1α were obtained. The alignment of the combined sequences consisted of a total of 1724 bp (658 bp of COI and 1066 bp of EF-1α genes, respectively), including 277 variable and 200 informative sites.

The pairwise P2K distances among the sequences were variable between genes. The ranges of sequence divergences for two loci and ingroup taxa are: COI (0–12.4%), EF-1α (0–5.0%). For COI, sequence divergence between conspecific individuals ranged from 0 to 0.6%; inter-specific genetic distances ranged from 3.6% to 12.4% with divergences among species averaging 7.9% (Table 2).

Table 2. Uncorrected pairwise genetic distances (Kimura 2-parameter) for the COI sequences of the genus *Scobura* species.

|		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	*S. cephaloides* 102																		
2	*S. cephaloides* 553	**0.003**																	
3	*S. coniata* 73	0.096	0.098																
4	*S. coniata* 472	0.096	0.098	**0.000**															
5	*S. hainana* 471	0.084	0.085	0.059	0.059														
6	*S. hainana* 487	0.084	0.085	0.059	0.059	**0.000**													
7	*S. hainana* 488	0.084	0.085	0.061	0.061	**0.002**	**0.002**												
8	*S. isota* 468	0.115	0.117	0.122	0.122	0.099	0.099	0.099											
9	*S. isota* 538	0.113	0.115	0.118	0.118	0.096	0.096	0.096	**0.006**										
10	*S. lyso* 465	0.087	0.089	0.061	0.061	0.039	0.039	0.039	0.105	0.101									
11	*S. lyso* 475	0.085	0.087	0.061	0.061	0.036	0.036	0.036	0.101	0.098	**0.003**								
12	*S. masutaroi* 300	0.092	0.096	0.059	0.059	0.056	0.056	0.056	0.107	0.103	0.052	0.052							
13	*S. masutaroi* 301	0.092	0.096	0.059	0.059	0.056	0.056	0.056	0.107	0.103	0.052	0.052	**0.000**						
14	*S. masutaroi* 303	0.092	0.096	0.059	0.059	0.056	0.056	0.056	0.109	0.105	0.052	0.052	**0.002**	**0.002**					
15	*S. parawoolletti* 116	0.094	0.097	0.089	0.089	0.087	0.087	0.087	0.112	0.108	0.084	0.084	0.084	0.084	0.084				
16	*S. stellata* 36	0.101	0.104	0.104	0.104	0.101	0.101	0.099	0.108	0.105	0.099	0.099	0.092	0.092	0.094	0.070			
17	*S. woolletti* 535	0.094	0.097	0.096	0.096	0.085	0.085	0.085	0.124	0.121	0.092	0.092	0.099	0.099	0.099	0.039	0.074		
18	*S. woolletti* 536	0.092	0.096	0.094	0.094	0.084	0.084	0.084	0.123	0.119	0.090	0.090	0.097	0.097	0.098	0.038	0.072	**0.002**	
19	*S. woolletti* 537	0.094	0.097	0.096	0.096	0.085	0.085	0.085	0.124	0.121	0.092	0.092	0.099	0.099	0.099	0.039	0.074	**0.000**	**0.002**
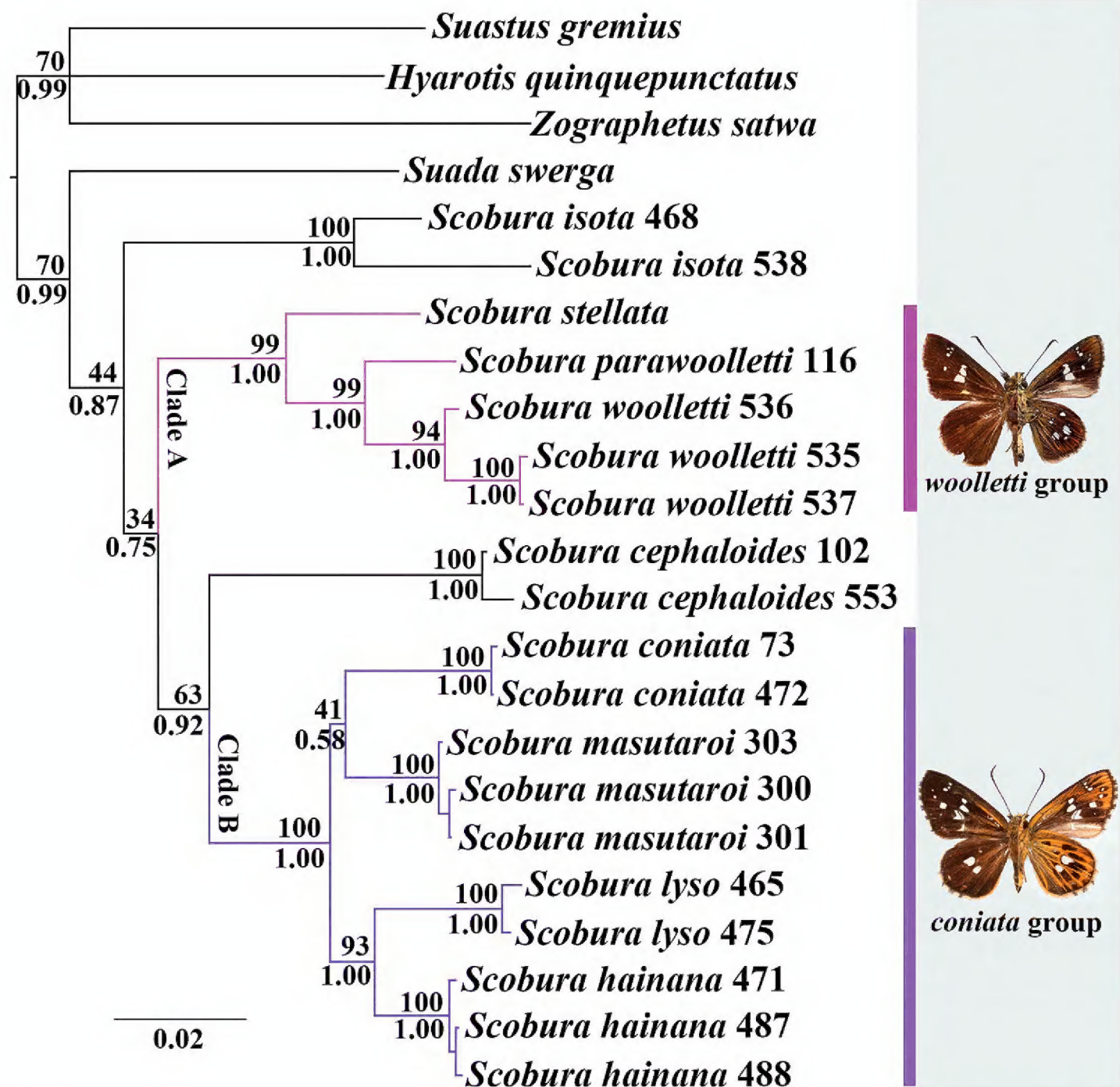
Figure 1. Majority-rule consensus tree from the Bayesian analysis (BI) of the concatenated COI and EF-1α sequences. Values at nodes represent the bootstrap support (BS) values of the maximum likelihood (ML) and the posterior probabilities (PP) of BI analyses, respectively (BP/PP).

Phylogenetic analyses

The two model-based analyses (BI and ML) revealed nearly identical topologies, differing mainly in branch support (Fig. 1). In both analyses, the monophyly of the genus *Scobura* is weakly supported (BP = 44, PP = 0.87). Within the genus, although support for the basal clades was low, the *Scobura* species included here are clearly distinguished from each other, and formed four clades: the *S. isota* clade (which only included two representative specimens), Clade A, the *S. cephaloides* clade (only with two representative specimens), and Clade B. Clade A is comprised by *S. stellata* + (*S. parawoolletti* + *S. woolletti*) and receive high bootstrap support and posterior probability (BP = 99, PP = 1.00). We hereafter called the clade *S. woolletti* group.
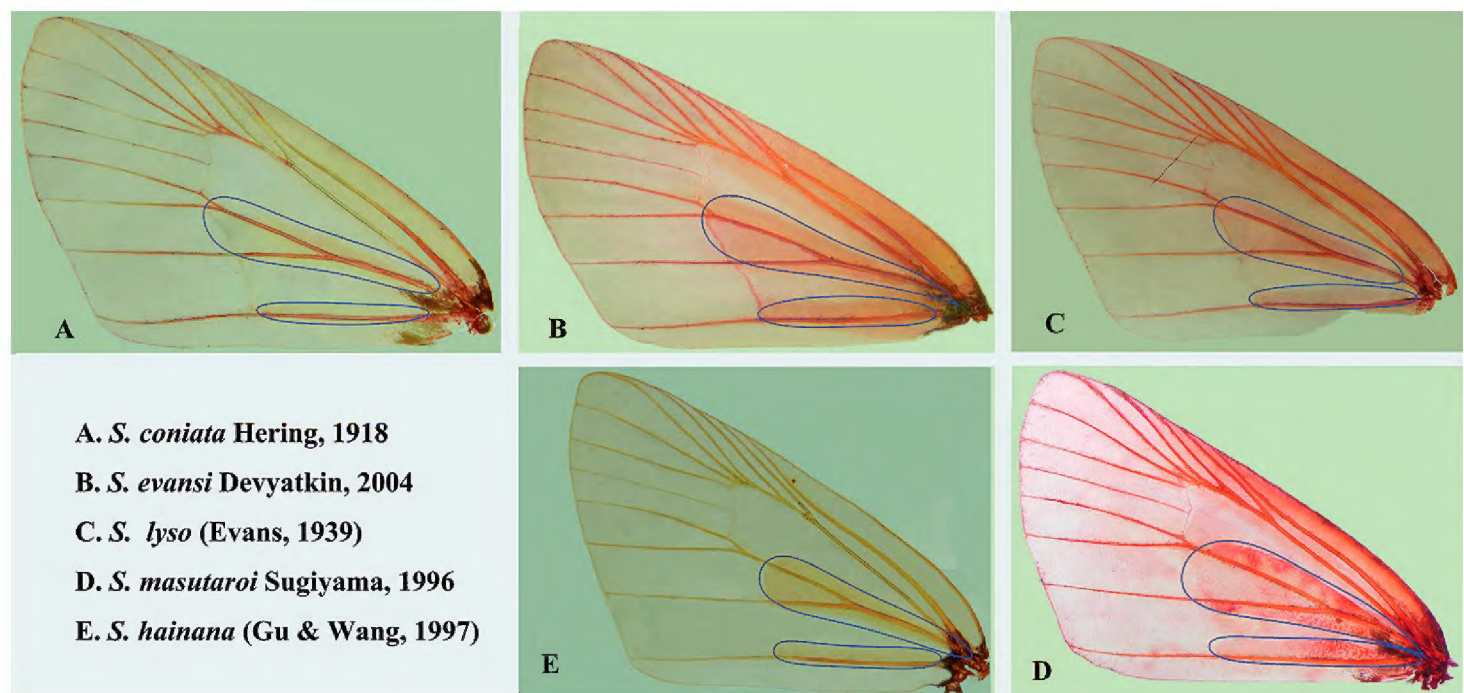
Figure 2. Male band of scent scales in the *S. coniata* group species.

Clade B is comprised by *S. masutaroi* and the representatives of *S. coniata* group (Devyatkin 2004): *S. coniata*, *S. lyso* and *S. hainana*, and the latter two are sister species with strong support (BP = 93, PP = 1.00). The monophyly of *S. coniata* group including *S. masutaroi* is strongly supported (BP = 100, PP = 1.00).

In all the analyses, *S. cephaloides* is sister to Clade B, with moderate support (BP = 63, PP = 0.92), whereas the relationships between *S. isota* and the other clades (Clade A, *S. cephaloides* and Clade B) remain unresolved.

Discussion

Although our phylogenetic analyses do not strongly support the monophyly of the genus *Scobura*, two strongly supported monophyletic groups within the genus are recognized: the *S. coniata* group and the *S. woolletti* group. The members of the *coniata* group share the following four morphological characters: 1) male band of scent scales on both sides of veins CuA_1 and CuA_2 and above 2A on the forewing (Fig. 2); 2) juxta U-shaped with two spine bearing arms, flat at base; 3) tegumen without socius; and 4) uncus thin and long. *S. masutaroi* is nested within this group. In our present analyses, two individuals (He 300, 301) of *masutaroi* from Nibashan, Sichuan (close to Dujiangyan, Sichuan, the type locality of *S. masutaroi*) and an individual (He303) from Jialingjiang, Fengxian, Shaanxi (the type locality of *S. mouchai*) are clearly grouped together with strong support values (BP = 100, PP = 1.00). Moreover, the pairwise P2K distances in COI between the species in the *S. coniata* group range from 3.3% to 6.1% with divergences between species averaging 4.5%, while divergence between individuals of *S. masutaroi* from Sichuan and Shaanxi province was 0.2%.

Based on the original description, distribution data, and the illustrations provided by Krajcik (2013), as well as our phylogenetic inferences, we conclude that *S. mouchai*

is identical to *S. masutaroi* and should be considered a junior synonym. The male genitalia are illustrated herein, and the female genitalia are described for the first time. On the basis of morphological study (Devyatkin, 2004), two other species, *S. phuongi* and *S. evani*, which are not included in the present study, likely also belong to this group.

A well-support clade comprised by *S. stellata*, *S. parawoolletti* and *S. woolletti* was recovered in all analyses. These species share the following three characters: 1) hindwing with white spots on underside but not on upperside; 2) socius slender and pointed at tip; and 3) juxta funnel-like, thin and long basally. The generic name *Mimambrix* Riley, 1923 was proposed with *Mimambrix woolletti* as the type species, but later synonymized by Evans (1949). We follow Evans' treatment and consider this clade as a species group within the genus *Scobura*. Based on morphological characters, the group also includes *S. tytleri* (Evans, 1914).

Taxonomic account

The key given by Fan et al. (2010) is modified to include *S. masutaroi*. The couplets leading to *S. masuataroi* only are included here. Couplets beyond 11 in the original increase their number by one.

3	Forewing upper side without spots in spaces M_3 or M_1 and M_2	4
–	Forewing upper side with spots in spaces M_1, M_2 and M_3	6
4	Forewing upper side without spots in spaces M_1 and M_3, hindwing under side: basal half yellow, distally ferruginous, with five small spots	***S. cephaloides***
–	Forewing upper side without spot in space M_3	5
5	Hindwing under side with a conspicuous rectangular white spot in space CuA_2	***S. cephala***
–	Hindwing under side without a conspicuous rectangular white spot in space CuA_2	***S. isota***
6	Hindwing upper side without spot in space CuA_1, under side with small white spots in spaces $Sc+R_1$, M_{1-2}, M_3 and cell	***S. eximia***
–	Hindwing upper side with the spot in space CuA_1	7
7	Forewing cell spots conjoined, subequal	8
–	Forewing cell spots separated, if conjoined, the lower spot much larger	9
8	Hindwing upper side hyaline spots white	***S.evansi***
–	Hindwing upper side hyaline spots yellow	***S.masutaroi***
9	Forewing upper side the spot in space CuA_2 triangular, and with a linear stigma crossing the spots in spaces CuA_1 and CuA_2	***S. coniata***
–	Forewing upper side the spot in space CuA_2 not as above	10
10	Forewing upper side the spot in space CuA_1 narrow, hindwing upper side without spot in space	***S. lyso***
–	Forewing upper side the spot in space CuA_1 broad	11

| 11 | Hindwing upper side spot in space M_3 tiny dot, forewing upper side cell spots cell spots conjoined..***S. hainana*** |
| – | Hindwing upper side spot in space M_3 significant, forewing upper side cell spots cell spots separated..***S. phuongi*** |

Scobura masutaroi Sugiyama, 1996
Fig. 3

Scobura masutaroi Sugiyama, 1996: 9 (Type locality: Dujiangyan, Sichuan, China)
Scobura mouchai Krajcik, 2013: 2, **syn. n.** (Type locality: Fengxian, Shaanxi, China)

Material examined. 1♂, 1♀, Nibashan, Rongjing, Sichuan, 26.VII.2009, Min Wang; 1♂, Jialingjiang, Fengxian, Shaanxi, 15.VII.2010, Min Wang.

Diagnosis. Forewing length 17–18 mm. This species is different from other species of *S. coniata* group in the appearance of the wing upper side: forewing with yellow streak in subcosta space basally, a big cell spots solid across cell, the spot in space CuA_2 yellow; hindwing with spots in spaces CuA_1 and M_1-M_2 yellow. Wing under side: forewing costal and submarginal spots yellow; hindwing all veins and submarginal spots from spaces $Sc+R_1$ to CuA_2 yellow; and all yellow submarginal spots conjoined both forewing and hindwing.
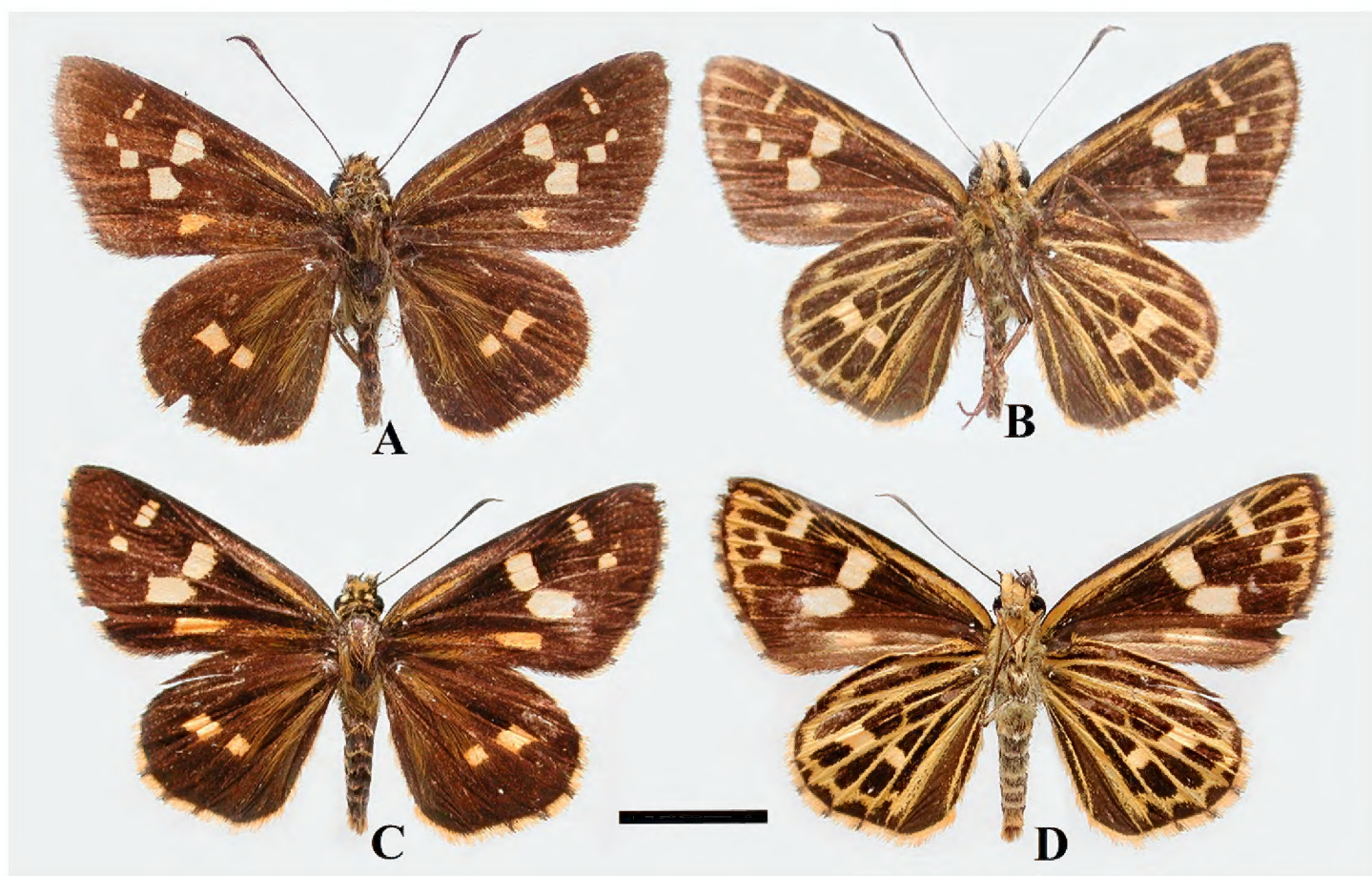

Figure 3. *Scobura masutaroi* Sugiyama, 1996 (Sichuan): **A, B** male **C, D** female; scale bar 10 mm.
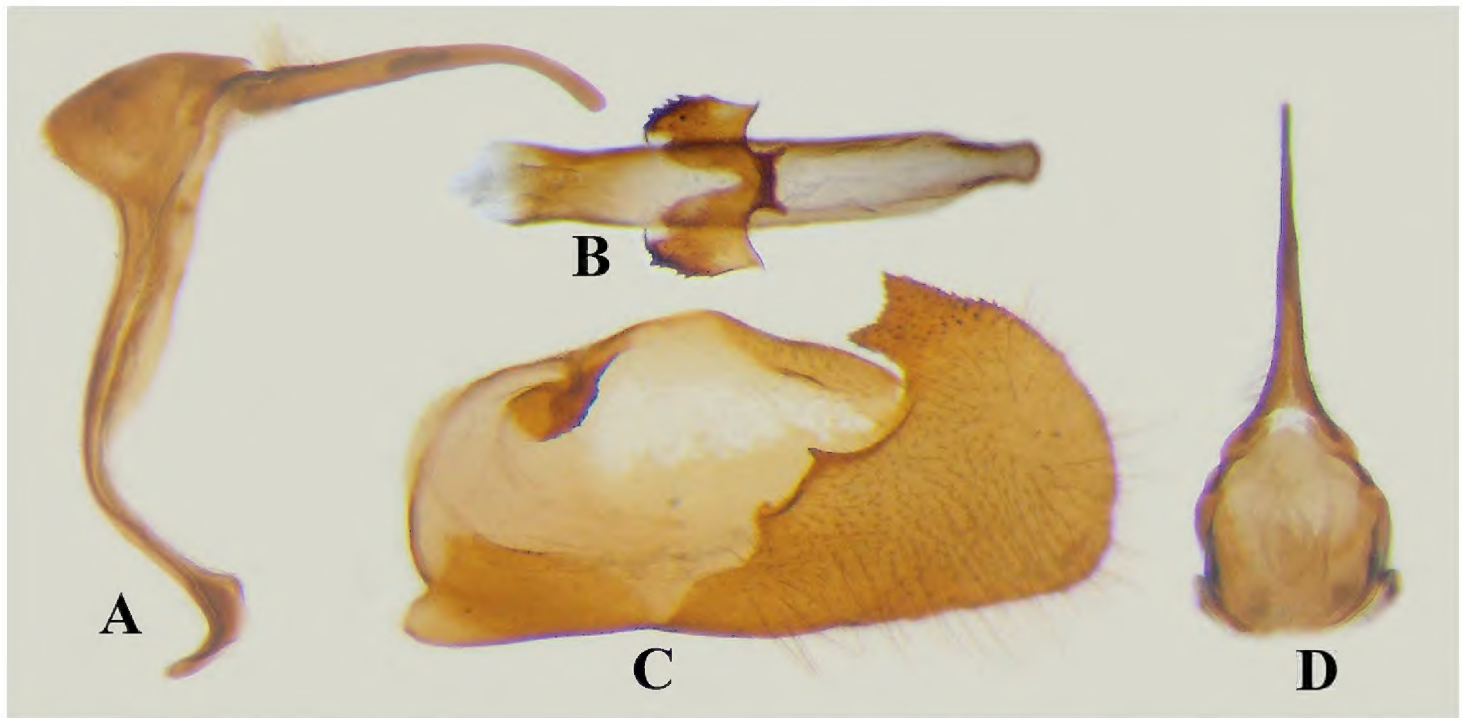
Figure 4. Male genitalia of *Scobura masutaroi* Sugiyama, 1996. (Sichuan). **A** Genitalia ring, lateral view; **B** aedeagus and juxta. **C** valva, inner view; **D** tegument, dorsal view.

Figure 5. Female genitalia of *Scobura masutaroi* Sugiyama, 1996 (Sichuan)

Description. Male genitalia (Fig. 4): Tegumen without socius, weakly rounded from lateral view; uncus slender and much longer than tegumen; valva with transtilla rounded and sclerotized with small spines, ventro-distal process irregularly shaped with outer edge rounded, inner edge uneven, and distal part rectangular with densely small spines; saccus short and broad; gnathos absent; juxta U-shaped with two arms with densely spines.

Female genitalia (Fig. 5): Papillae anales rectangular, covered with setae; anterior lamella U-shaped with sclerotization; posterior lamella triangular with upper margin arched; ductus bursae membranous and short; copulatrix bursa elongate, membranous.

Distribution. China (Sichuan, Shaanxi).

Acknowledgements

References

Devyatkin AL (2004) Taxonomic studies on Oriental Hesperiidae, 1. A revision of the *Scobura coniata* Hering, 1918-group. Atalanta 35(1/2): 57–66.

Evans WH (1949) A catalogue of the Hesperiidae from Europe, Asia & Australia in the British Museum (Natural History). The British Museum, London, England, United Kingdom, 502 pp. https://doi.org/10.5962/bhl.title.105941

Fan XL, Chiba H, Wang M (2010) The genus *Scobura* Elwes & Edwards, 1897 from China, with descriptions of two new species (Lepidoptera: Hesperiidae). Zootaxa 2490: 1–15.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular marine biology and biotechnology 3: 294–299.

Gu MB, Chen PZ (1997) Butterflies in Hainan Island. China Forestry Publishing House. Beijing, China, 337 pp.

Krajcik M (2013) Description new Scobura Elwes and Edwards from Shaanxi Province and notes on the genus Ampittia Moore in China (Lepidoptera, Hesperioidea, hesperiinae). ANMMA 10(57): 1–3.

Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for Inference of Large Phylogenetic Trees. In: SC10 Workshop on Gateway Computing Environments (GCE10). https://doi.org/10.1109/GCE.2010.5676129

Monteiro A, Pierce NE (2001) Phylogeny of *Bicyclus* (Lepidoptera: Nymphalidae) Inferred from COI, COII, and EF-1α Gene Sequences. Molecular Phylogentic Evolution 18(2): 264–281. https://doi.org/10.1006/mpev.2000.0872

Rambaut A, Suchard MA, Xie D, Drummond AJ (2014) Tracer v1.6. http://beast.bio.ed.ac.uk/Tracer

Riley ND (1923) New Rhopalocera from Borneo. Entomologist 56: 35–38.

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice Across a Large Model Space. Systematic Biology 61: 539–542. https://doi.org/10.1093/sysbio/sys029

Stamatakis A (2014) RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. Bioinformatics. https://doi.org/10.1093/bioinformatics/btu033

Sugiyama H (1996) New Butterflies from Western China (IV). Pallarge 5: 1–11.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution 30(12): 2725–2729. https://doi.org/10.1093/molbev/mst197

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 24: 4876–4882. https://doi.org/10.1093/nar/25.24.4876

Wang HS, Xiong W, Wang M (2011) Two new species of the genus *Longipenis* (Lepidoptera: Lecithoceridae) from China. Florida Entomology 93: 352–356. https://doi.org/10.1653/024.093.0305